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Differential role for PAK1 and PAK4 during the invadopodia lifecycle

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Abstract

PAK1 and PAK4 are members of the p-21 activated kinase family of serine/threonine kinases. PAK1 has previously been implicated in both the formation and disassembly of invasive cell protrusions, termed invadopodia. We recently reported a novel role for PAK4 during invadopodia maturation and confirmed a specific role for PAK1 in invadopodia formation; findings we will review here. Moreover, we found that PAK4 induction of maturation is delivered via interaction with the RhoA regulator PDZRho-GEF. We can now reveal that loss of PAK4 expression leads to changes in invadopodia dynamics. Ultimately we propose that PAK4 but not PAK1 is a key mediator of RhoA activity and provide further evidence that modulation of PAK4 expression levels leads to changes in RhoA activity.

As melanoma cells move through complex tissue there is a regulated rearrangement of the actin cytoskeleton a process thought to be co-ordinated by Rho family GTPases¹. Moreover, during tissue migration cancer cells need to navigate the 3D architecture of the densely packed stroma. To achieve efficient migration these cells are thought to employ protease-secreting invasive protrusions rich in actin, termed invadopodia². These invadopodial structures are now widely accepted to occur both *in vitro* and *in vivo* and are also thought to be critical for intravasation of the vasculature^{3, 4}.

Kinase activity forms a vital part of cytoskeletal dynamics including the regulation of invadopodia⁵. The p-21 activated kinase (PAK) family of serine/threonine kinases are known effectors of Rho GTPases Rac and Cdc42 that control cytoskeletal dynamics and cell movement⁶. The human PAK family consists of six isoforms, which are separated into two groups according to their sequence and structural homology: group I, containing PAKs 1-3; and group II, containing PAKs 4-6⁶.

The overexpression of PAKs is found in a wide variety of cancer tissue types and is often associated with an increase in invasive potential and poor prognosis⁶. PAK1 has been shown to localise to invadopodia⁷, however, studies investigating the specific function of this protein in invadopodia formation/function have yielded conflicting results. Previous studies in melanoma provide evidence that PAK1 is important for the formation of invadopodia via the phosphorylation of cortactin at Ser113⁸. However, a more recent study in breast cancer suggests that PAK1 (once again via the phosphorylation of cortactin at Ser113), promotes the disassembly of invadopodia protrusions; with a depletion of PAK1 leading to an increase in matrix degradation⁷. Additionally, several investigations have been conducted into the function of PAK1 in the invadopodia-like protrusion, podosomes. These studies agree that reduced PAK1 activity results in a decrease in podosome formation⁹ and increased PAK1 expression enhanced the formation of this protrusion¹⁰. Despite the controversy PAK1 is clearly associated with invadopodia dynamics, in contrast, the role of related family member PAK4 had not been previously explored. Although, it had been shown that PAK4 was localised to podosomes and that depletion of PAK4

reduced podosome number¹¹. We were therefore interested to test if PAK4 also played a functional role in invadopodia.

Whilst PAK1 and PAK4 are structurally distinct and activated differently⁶ they share a plethora of overlapping substrates and the unique signalling pathways that may drive invasive potential during tumourigenesis have not been clearly defined¹². Research into these potential differences could help guide the further development of therapeutic drugs. Currently, pharmaceutical companies are focused on developing group or isoform specific inhibitors¹³⁻¹⁵. Therefore, data indicating whether both groups contribute to invasion and metastasis in the same way, may determine whether the use of pan-selective inhibitors is more beneficial than isoform selective inhibitors in treating some cancer types. Despite the difficulties in separating PAK1 and PAK4 functions mouse knockout (KO) phenotypes suggest that at least for PAK4 there are isoform specific functions as PAK4 KO mice are embryonically lethal whilst PAK1 KO mice remain viable and fertile⁶.

We sought to address this issue of isoform specificity by performing the same quantitative invasion assays on cells depleted of either PAK1 or PAK4 expression¹⁶. From this work we were able to provide further evidence that the predominant role of PAK1, at least in melanoma, is to drive invadopodia formation rather than disassembly. Using a gelatin degradation assay we were able to show that cells depleted of PAK1 expression do not initiate actin puncta; indicative of invadopodia formation and there is no subsequent degradation of the underlying matrix. However we observed a different phenotype in PAK4 depleted cells. These cells were able to form actin puncta but were then unable to degrade the underlying matrix. Thus by systematically testing PAK1 and PAK4 depletion in the same cell lines, using the same functional assay, we were able to identify a differential function. Where PAK1 activity is focussed towards the early stages of invadopodia formation and PAK4 activity restricted to the later maturation stages (**Figure 1A**). The formation of puncta but loss of degradation placed PAK4 at the maturation phase of the invadopodia lifecycle and we detected a loss of membrane type-1-matrix metalloproteinase (MT1-MMP) localisation. To further explore the difference between the two PAK isoforms we performed live cell imaging to ascertain the lifetime of invadopodia formation in the depleted cells. To directly image invadopodial dynamics stable control shRNA,

PAK1shRNA and PAK4shRNA A-375M2 cells were infected with lentivirus to stably express LifeAct-mRFP. These cells were plated onto gelatin and immediately treated with GM6001 to inhibit invadopodia formation. The inhibitor was then removed 1h prior to imaging to synchronise the population and maximise data acquisition. Invadopodia assembly and disassembly rates were calculated by measuring the fluorescence intensity of the actin rich invadopodia. As expected the actin puncta that we could detect in PAK1 depleted cells were extremely unstable and accurate quantification of lifetimes was not achievable. However in contrast to PAK1 depleted cells the actin puncta in PAK4 depleted cells exhibited a longer lifetime than control cells (Figure 1B and C). Thus, the invadopodia formed in PAK4 depleted cells were more persistent and fail to turnover at the expected rate (**Figure 1B and C**). These data, further support a role for PAK4 activity at the later stages where disassembly is considered the final requirement during the invadopodia lifecycle.

These observations coupled with our finding that both PAK1 and PAK4 kinase activity is required in the invadopodia, led us to explore the possibility that PAK1 and PAK4 may have different substrates in the invadopodia. Whilst PAK1 phosphorylation of cortactin is established during formation⁸, PAK4 activity had not been previously reported in invadopodia. We have now identified PDZ-RhoGEF as a PAK4 binding protein, already known to be phosphorylated and inactivated by PAK4¹⁷, with functional significance in invadopodia dynamics. The novel function of this PAK4/PDZ-RhoGEF pathway in invadopodia dynamics was confirmed by the use of a dominant negative mutant of PDZ-RhoGEF which could bind, but not activate Rho^{18, 19}. The expression of this mutant in cells with depleted PAK4 protein resulted in the rescue of invadopodia formation and degradation back to levels seen in wildtype cells. Furthermore, the expression of wildtype PDZ-RhoGEF mimicked a PAK4 depletion phenotype, reducing both the percentage of cells with invadopodia and the invadopodia induced matrix degradation. PDZ-RhoGEF, along with PAK4 was found to be localised to invadopodia supporting the suggestion that the proposed signalling pathway occurs within these protrusions. This localisation also suggests that RhoA activation is likely required at some stage during the lifecycle of the protrusion (low levels of Rho activity in invadopodia can be easily achieved by the localisation of PDZ-RhoGEF away from the protrusion). Our hypothesis is that

PAK4 phosphorylates and inactivates PDZ-RhoGEF which in turn suppresses RhoA activity and allows the invadopodia to mature. PDZ-RhoGEF is known to be specific for Rho, with no binding to Cdc42 or Rac1. Moreover this GEF preferentially activates RhoA, over RhoB and RhoC²⁰. Investigations into the function of RhoA in invadopodia have yielded contradictory results. Some studies have suggested that reduced RhoA expression decreases invadopodia formation and degradation²¹, while others have found no effect on invadopodia degradation when RhoA activation is inhibited or activated²². However, recent studies suppressing RhoA activity promoted invadopodia formation²³ and work with podosomes have indicated that RhoA inactivation is required for podosome formation²⁴ whilst the constitutive activation of this protein reduces podosome formation^{24, 25}. It has been suggested that a balance of RhoA activation and inactivation is important for podosome function with both the constitutive activation and reduction of RhoA activity resulting in reduced podosome degradation²⁶. Our findings suggest that the same may be true for invadopodia function and provide additional evidence that the control of RhoA activity is important in invadopodia function. To date, the mechanism by which the PAK4/PDZ-RhoGEF/RhoA pathway functions in invadopodia is unknown. RhoA plays a key role in the actin cytoskeletal dynamics and actomyosin contractility which is important in the formation of protrusions such as invadopodia²⁷. Therefore, one potential mechanism by which the inhibition of RhoA brought about by the PAK4/PDZ-RhoGEF/RhoA pathway can function at invadopodia, is through a reduction in the contractile force exerted on the protrusion to allow for the extension of the membrane. Furthermore, with RhoA activation being important for focal adhesions (incorporation of stress fibres), which is often associated with invadopodia dissolution, RhoA inactivation at invadopodia may tip the balance away from invadopodia formation towards disassembly as RhoA activity is redirected towards the formation of focal adhesions. It will be interesting to explore further how levels of RhoA activity correlate with invadopodia dynamics.

We were able to show both in our recent melanoma work and previous work²⁸ with prostate cancer cells that depletion of PAK4 leads to an increase in RhoA activity measured both by activity pulldown assays and FRET biosensors. There is a particular level of complexity surrounding the role of PAK1/PAK4 in regulation of RhoA activity. PAK4 is purported to contain a GEF interacting domain (GID)⁶ not

found in PAK1, however both PAK1 and PAK4 have been reported to inhibit RhoA activator, GEF-H1²⁹⁻³¹. Phosphorylation of GEF-H1 by PAK1 or PAK4 at serine 885 initiates a 14-3-3 binding event that is thought to sequester GEF-H1 on microtubules and inhibit activation of RhoA. Indeed, our previous work demonstrated that PAK4 depletion can elevate the level of RhoA activity²⁸ whilst concomitantly decreasing GEF-H1 S885 phosphorylation^{32, 33}. In contrast RhoA activation has not been observed in PAK1 depleted cells³⁴. Moreover these PAK1:GEF-H1 studies were not conducted in melanoma cell lines and in our study we did not see modulation of GEF-H1 serine phosphorylation in PAK1 nor PAK4 depleted cells. It should be noted that PAK2 and PKA can also phosphorylate GEF-H1 at this site^{35, 36}. In our recent study we also did not find a change in RhoA activity in PAK1 depleted cells using the FRET biosensors.

To further investigate the relationship between PAK4 and regulation of RhoA we have extended our monitoring of the prominence of actin stress fibres in our PAK4 depleted cells as a readout for RhoA activity. Control and PAK4 depleted WM-115 and A-375M2 cells were seeded onto glass or gelatin coated coverslips allowed to adhere for 3 h and then fixed and stained for F-actin. The cells were imaged (**Figure 2A**) and the images quantified for the presence of prominent F-actin stress fibres. Interestingly we found that on both glass and gelatin PAK4 depleted cells display an increase in prominent actin stress fibres (**Figure 2B**). To confirm that this increase in actin stress fibres is specific to loss of PAK4 expression we also expressed GFP-alone or GFP-PAK4r (resistant to the PAK4 specific siRNA sequence) in PAK4siRNA treated cells prior to plating. All cells were fixed and stained for F-actin and the presence of prominent actin stress fibres quantified. Importantly we found that PAK4 depleted cells and PAK4 depleted cells expressing GFP-alone exhibited higher levels of actin stress fibres compared to control but that PAK4 depleted cells expressing siRNA resistant GFP-PAK4r returned to control levels (**Figure 2C and D**).

In conclusion, using our systematic approach we have demonstrated that both PAK1 and PAK4 play an important role in melanoma cell invasion (**Figure 3**) but have distinct pathways in invadopodia function; with PAK4 promoting maturation and/or degradation through the localised inhibition of PDZ-RhoGEF. Taken together our

recent study¹⁶ and previous work²⁸ strongly points to a significant regulatory role for PAK4 in the RhoA pathway via its interaction with multiple RhoA GEFs. We believe that this is a distinction between PAK1 and PAK4 that is likely to play out in multiple cells and processes.

Methods/Materials

Cell culture

The melanoma cell line A-375M2 was grown in Dulbecco's modified eagle's medium: nutrient F-12 ham (DMEM F-12) (containing L-glutamine), and the WM-115 cell line was grown in minimum essential medium (MEM) (containing L-glutamine). All the growth media were supplemented with 10% foetal bovine serum (FBS), penicillin and streptomycin sulphate. A-375M2 cells were transiently transfected using Lipofectamine® 2000 transfection reagent, according to the manufacturer's instructions. Where indicated ethanol washed coverslips were coated with gelatin and fixed with glutaraldehyde.

siRNA/constructs

Oligonucleotides (Dharmacon, UK) were transiently transfected at a concentration of 25nM using HiPerFect transfection reagent (Qiagen), according to the manufacturer's instructions. Control siRNA oligonucleotide (AATTCTCCGAACGTGTCACGT) PAK4 Oligo 1 siRNA oligonucleotide (GGTGAACATGTATGAGTGT) PAK4 Oligo 2 siRNA oligonucleotide (CGAGAATGTGGTGGAGATGTA). GFP-PAK4r was constructed by site-directed mutagenesis, according to the manufacturer's instructions, using the QuikChange Multisuite II kit (Stratagene). pENTR-PAK4³⁷ was used as template DNA for site-directed mutagenesis reactions. GFP Alone vector was purchased from Clontech.

Immunofluorescence

Cells were fixed with 4% (w/v) paraformaldehyde (PFA) and permeabilised using 0.2% (v/v) triton X-100 and then washed with PBS. Non-specific binding was blocked by 3% BSA. Coverslips were incubated for 2 hours with the primary antibody and then washed with PBS. Cells were incubated for 1 hour with secondary antibody and fluorophore conjugated phalloidin (Invitrogen). Coverslips were then washed with PBS and mounted using Fluorsave™ reagent. Images were acquired using an Olympus IX71 microscope.

Invadopodia lifetime Imaging

Stable control shRNA, PAK1shRNA and PAK4shRNA A-375M2 cells were infected with lentivirus to stably express LifeAct-mRFP (kind gift from Prof. Maddy Parsons). Stable control and knockdown cells expressing LifeAct-mRFP were seeded on gelatin (not fluorophore conjugated) and treated with 25uM GM6001 (VWR) overnight to inhibit invadopodia formation. The inhibitor was removed 1h before imaging by replacing media. Images were taken at 15 second intervals over 15 mins using a Nikon A1R confocal microscope. Invadopodia assembly and disassembly rates were calculated by measuring the fluorescence intensity of the actin rich invadopodia using imageJ (Intensity vs. time plot). Data values were exported and visualised in Excel and invadopodia assembly (from lowest data point to max peak) and disassembly (max peak to the following lowest data point) were calculated.

Disclosure of interest

The authors report no conflict of interest

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Figure Legends

Figure 1: PAK4 activity is restricted to the maturation phase of invadopodia lifecycle.

(A) Possible functions for PAK1 and PAK4 in the invadopodia lifecycle. PAK1 plays a role in the formation of nascent invadopodia. PAK4 functions in the maturation/degradation stage of the invadopodia lifecycle. **(B) and (C)** Lifetime imaging of A-375M2 cells with depleted PAK4 expression. Lifeact-mRFP transfected cells were plated on gelatin and images were taken at 15 second intervals over 15 mins. Representative images are shown in 75 second intervals for each condition. Significance was calculated to wildtype cells. Data are mean values \pm S.E.M. of 15 cells, over 3 independent experiments; * = $P < 0.05$.

Figure 2 PAK4 specifically influences RhoA activity

Cells were plated on glass or gelatin matrix coated coverslips 4 days post-transfection of siRNA oligonucleotides to reduce PAK4 expression. **(A)** Representative images of WM-115 and A-375M2 cells on glass and gelatin for each condition. Scale bar = 10 μ m **(B)** Quantification of the percentage of cells with prominent actin fibres. Significance was calculated to wildtype and control cells transfected with non-specific siRNA. Data are mean values \pm S.E.M. of 150 cells, over 3 independent experiments; * = $P < 0.05$. Control = cells transfected with non-specific siRNA. **(C)** Cells were plated in 6 well plates and transfected with siRNA oligonucleotides to reduce PAK4 expression. Two days post-transfection the cells were transfected with GFP alone or GFPPAK4r constructs. After 48 hrs, these cells were seeded on glass coverslips and incubated overnight, fixed and stained for F-actin. Quantification of the percentage of cells with prominent actin fibres. Significance was calculated to control cells transfected with non-specific siRNA. Data are mean values \pm S.E.M. of 150 cells, over 3 independent experiments; * = $P < 0.05$. Control = cells transfected with non-specific siRNA. **(D)** Representative images of A-375M2 cells for each condition. Scale bar = 10 μ m

Figure 3: PAK1 and PAK4 differential function in invadopodia dynamics.

PAK4 localises to the invadopodia and inhibits the function of PDZ-RhoGEF. This in turn prevents the activation of RhoA to promote invadopodia maturation and turnover. This may be through the inhibition of membrane contraction. Active RhoA may

function in invadopodia to retract the protrusion during disassembly. Speculative pathways are labelled in red. PAK1 does not signal through PDZ-RhoGEF in invadopodia. It may function via the phosphorylation of cortactin or another substrate.

Figure 1

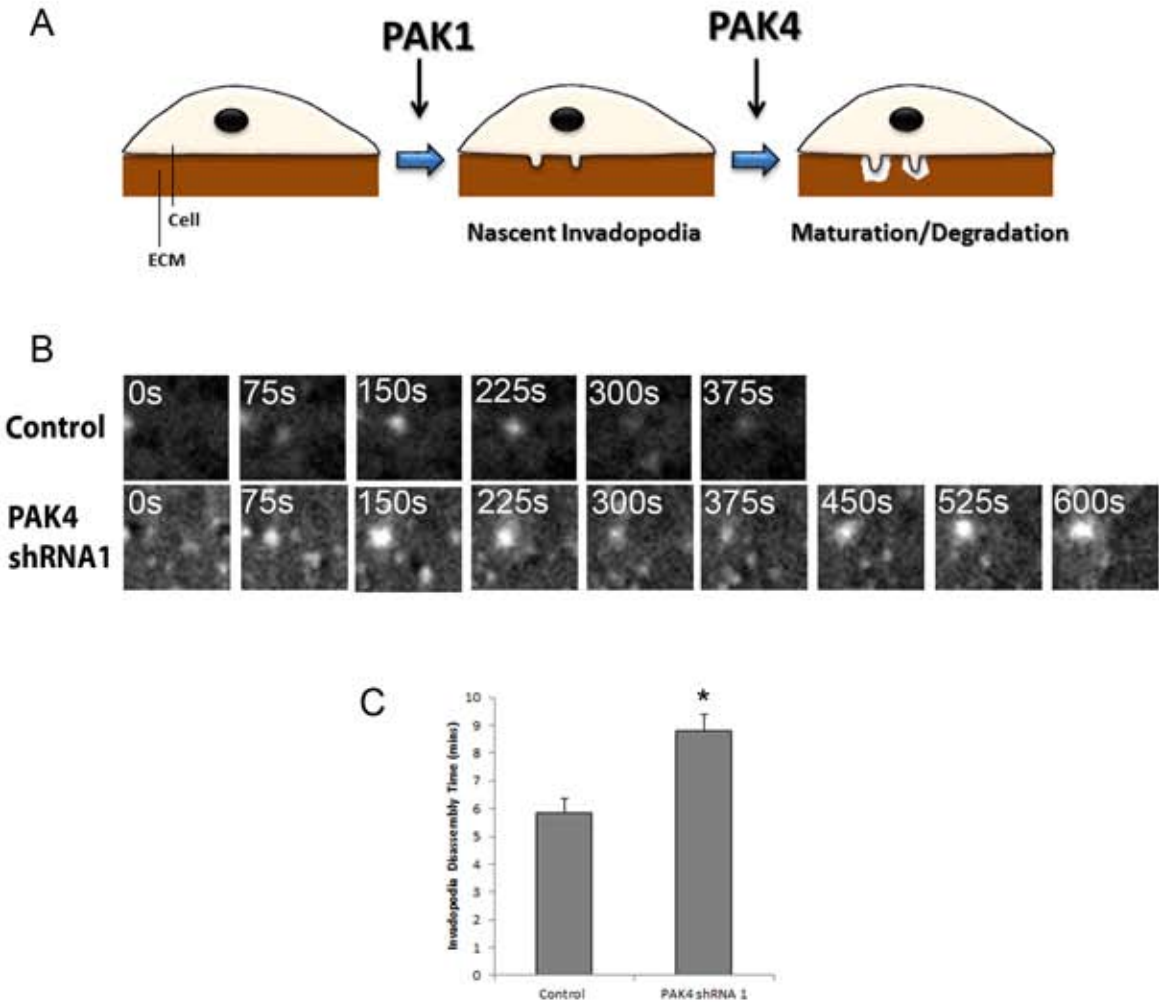


Figure 2

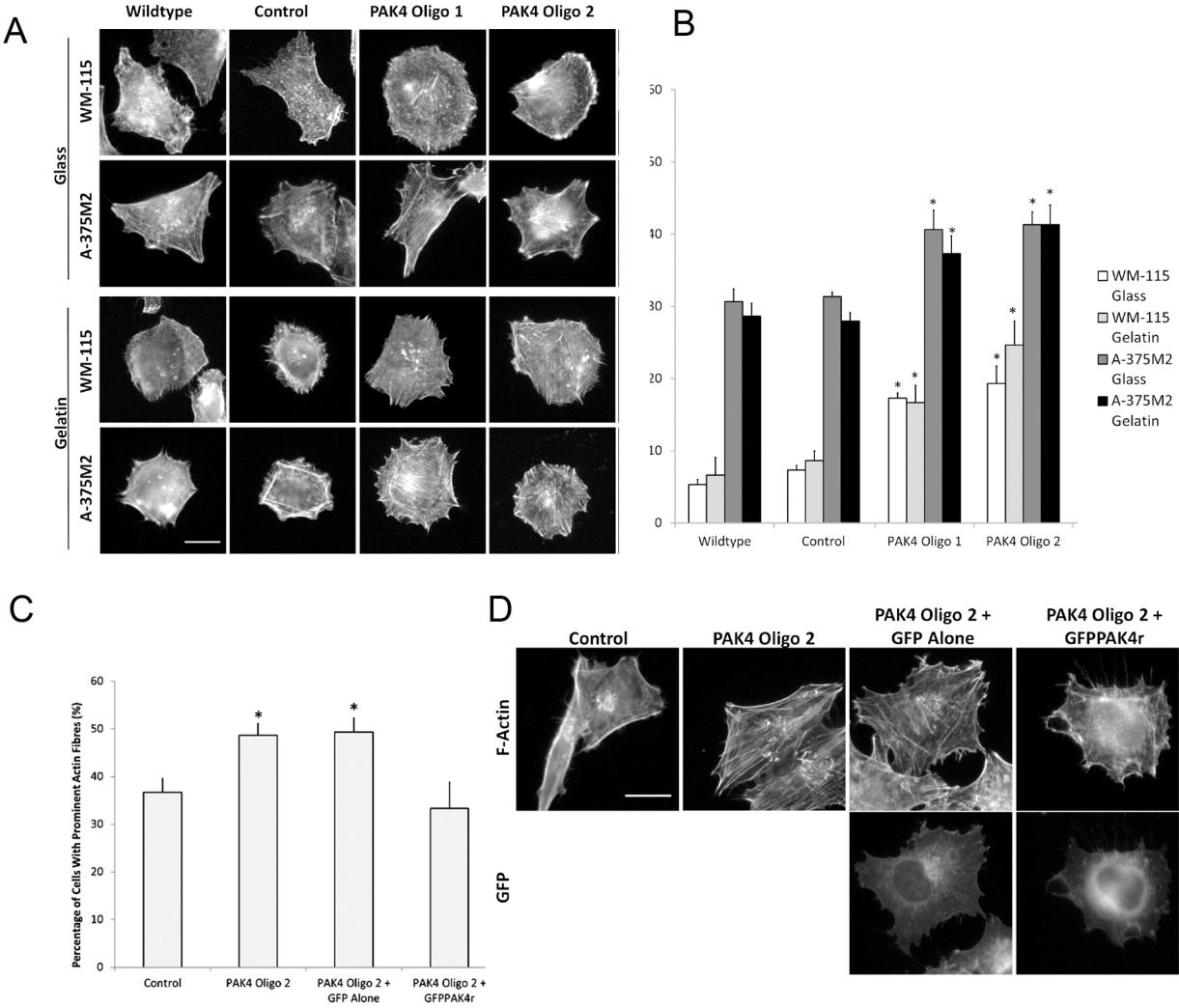


Figure 3

